Molecular Pathways: Targeting the CXCR4–CXCL12 Axis—Untapped Potential in the Tumor Microenvironment

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Running Title: CXCR4 in Tumor Microenvironment

Disclosure of Potential Conflicts of Interest

S. Scala is listed as a co-inventor on a patent, which is co-owned by Istituto Nazionale per lo Studio e la Cura dei Tumori and Consiglio Nazionale delle Ricerche, related to cyclic peptides binding CXCR4 receptor and relative medical and diagnostic uses. No other potential conflicts of interest were disclosed.
Abstract

Evidence suggests the CXC-Chemokine Receptor-4 pathway, plays a role in cancer cell homing and metastasis, and thus represents a potential target for cancer therapy. The homeostatic microenvironment chemokine, CXCL12, binds the CXCR4 and CXCR7 receptors, activating divergent signals on multiple pathways such as ERK1/2, p38, SAPK/JNK, AKT, mTOR and the Bruton Tyrosine kinase (BTK). An activating mutation in CXCR4 is responsible for a rare disease, WHIM syndrome, and dominant CXCR4 mutations have also been reported in Waldenstrom Macroglobulinemia (WM). The CXCR4/CXCL12 axis regulates the hematopoietic stem cell niche - a property that has led to the approval of the CXCR4 antagonist, plerixafor (AMD3100), for mobilization of hematopoietic precursors. In preclinical models plerixafor has shown anti-metastatic potential in vivo, offering proof of concept. Other antagonists are in preclinical and clinical development. Recent evidence demonstrates that inhibiting CXCR4 signaling restores sensitivity to CTLA4 and PD-1 checkpoints inhibitors, creating new line for investigation. Targeting the CXCR4-CXCL12 axis thus offers the possibility of affecting CXCR4-expressing primary tumor cells, modulating the immune response or synergizing with other targeted anti-cancer therapies.
Background

Chemokines are small chemo-attractant cytokines that are expressed in discrete anatomical locations. In adult vertebrates, chemokines are essential for proper lymphoid organ architecture and for leukocyte trafficking (1). Chemokines are classified according to their conserved N-terminal cysteine residues (C) that form the first disulfide bond. These residues can be adjacent (CC) or separated by amino acid(s) (CXC, CX3C). Forty-seven chemokines have been identified - 27 CC and 17 CXC chemokines as well as a single CX3C and 2 single C chemokines (1,2). Chemokines act on chemokine receptors (CKR), members of the seven transmembrane domain G protein coupled receptor (GPCR) superfamily. Classically, one of the chemokine receptor intracellular loops interacts with heterotrimeric, pertussis toxin-sensitive G proteins called Gαi, initiating a cascade of signal transduction events in response to ligand binding. In addition, chemokine receptors can signal through non-G protein-mediated pathways or even through other G-protein subtypes (3). There are also atypical chemokine receptors, (ACKR), which do not mediate conventional signaling and do not elicit directional migration (4,5); the CXCL12 – CXCR4 axis will be the focus of this Molecular Pathways review.

Encoded on chromosome 2q21, CXCR4 is an evolutionarily highly conserved GPCR expressed on monocytes, B cells and naive T cells in the peripheral blood. Human CXCR4 was originally identified as a receptor for CXCL12 by screening chemokine receptor orphan genes for their ability to induce intracellular Ca\(^{2+}\) in response to human CXCL12. Its ligand, CXCL12 is a homeostatic chemokine, which controls hematopoietic cell trafficking, adhesion, immune surveillance and development. The amino-terminal domain of CXCL12 binds the second extracellular loop of CXCR4 and activates downstream signaling pathways. The third intracellular loop of CXCR4 is necessary for Gαi-dependent signaling, and intracellular loops 2 and 3 as well as the C terminus of CXCR4 are required for chemotaxis (6,7).
CXCL12 binding to CXCR4 triggers multiple signal transduction pathways able to regulate intracellular calcium flux, chemotaxis, transcription and cell survival (8). CXCL12 binding promotes a three-dimensional CXCR4 conformation favoring Gαi protein dissociation into α- and βγ-subunits, (8,9). In turn, different subtypes of the α subunit impart different signals. Gαi subunits inhibit cAMP formation via inhibition of adenylyl cyclase activity and the αq-subunits activate phospholipase C (PLC)-β, generating diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3), which controls the release of intracellular Ca²⁺. While inhibiting adenyl cyclase, the Gai subunits activate the NF-kB, JAK/STAT, and PI3K-AKT pathways as well as mTOR, and the JNK/p38 MAPKs regulating cell survival, proliferation, and chemotaxis. Recent studies have shown CXCR4 signaling through mTOR in pancreatic cancer, gastric cancer and T-cell leukemia cells (10-13). In human renal cancer cells, CXCL12 induces phosphorylation of the specific mTOR targets, P70S6K and 4EBP1 (14) and CXCR4 and mTOR inhibitors have been reported to impair human renal cancer migration (14) (Fig. 1). Unlike the α subunits, βγ dimer subunits promote RAS-mediated MAPK signaling thereby regulating cell proliferation and chemotaxis (6). Finally, in addition to these classical signaling pathways, CXCR4 triggers Bruton Tyrosine Kinase (BTK) phosphorylation and downstream MAPK in mantle cell lymphoma and primary acute myeloid leukemia (AML) blasts, suggesting a potential interaction of CXCR4 on BTK and a potential for concomitant CXCR4 and BTK inhibition, the latter possibility raised by the availability of a recently approved inhibitor of BTK, ibrutinib (15).

CXCL12 binding to CXCR4 also causes CXCR4 desensitization, manifested as uncoupling from G-proteins by GPCR kinase (GRK)-dependent phosphorylation and subsequent interaction of CXCR4 with β-arrestin, which mediates internalization of the receptor (16) (Fig. 1). Upon internalization, CXCR4 is targeted for lysosomal degradation (9, 16). This requires agonist-induced ubiquitination of the carboxyl terminal tail lysine residues (16).
Recently, CXCR7 was also found to be a high affinity receptor for CXCL12 and for CXCL11 (4, 17, 18). CXCR7, renamed as ACKR3, belongs to the atypical chemokine receptor group (ACKR) of proteins, which do not mediate conventional signaling and do not elicit directional migration. The highly conserved DRYLAIV domain, which controls G-protein binding and activation in CXCR4, is replaced by DRYLSIT in the CXCR7 protein. Thus, typical chemokine responses mediated by G protein activity are not generated after ligand binding. However, recent evidence suggests that CXCR7 activates intracellular signaling pathways, including the AKT, MAPK and JAK/STAT3 pathways (18). CXCR7 is also regulated by ubiquitination, but in contrast to CXCR4, CXCR7 is constitutively ubiquitinated and agonist-activation induces its de-ubiquitination. Upon agonist binding, CXCR7 is rapidly internalized via a β-arrestin-dependent pathway and recycled to the cell surface. CXCR7 itself is not degraded, but rather it delivers bound chemokine to lysosomes for degradation (16). β-arrestin recruitment to the CXCR4/CXCR7 complex enhances downstream β-arrestin-dependent cell signaling (ERK1/2, p38, SAPK/JNK), which induces cell migration in response to CXCL12 (11, 13, 18, 19).

Clinical–Translational Advances

The CXCR4 antagonist AMD3100 is the most studied among the agents that inhibit CXCL12/CXCR4 signaling. AMD3100 was initially studied as an anti-HIV agent and then it was discovered that this compound increases white blood cell counts in the blood and is able to mobilize stem cells from the bone marrow (20, 21). Cell migration in and out of the bone marrow follows opposite chemokine signals, which implies that chemokines regulate the numbers of circulating granulocytes and monocytes. CXCL12 is the predominant signal that retains CXCR4+ hematopoietic stem cells (HSCs) inside the bone marrow (21). Plerixafor (previously AMD3100) is available clinically, having been developed as a mobilizing agent for hematopoietic precursors. The FDA approved plerixafor in 2008 for “use in combination with granulocyte-colony stimulating...
factor (G-CSF) to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin lymphoma (NHL) and multiple myeloma (MM)” (20, 22).

A clinical indication for CXCR4 antagonists has emerged recently in the rare human WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis). WHIM syndrome is associated with germ line dominant mutations in the C terminus of CXCR4, which result in a truncation of the receptor (23). This blocks receptor internalization, determining persistent CXCR4 activation and bone marrow myeloid cell retention, an outcome known as myelokathexis, the retention and apoptosis of mature neutrophils in the bone marrow (24). Two Phase I trials showed that plerixafor was able to safely and rapidly increase absolute lymphocyte, monocyte, and neutrophil counts in the peripheral blood of WHIM syndrome patients in a dose-dependent manner for 1 to 2 weeks (25). Another Phase I study demonstrated a durable increase in circulating leukocytes, fewer infections and improvement in warts in combination with topical imiquimod in three patients with WHIM syndrome who self-injected a low dose of plerixafor (4% to 8% of the FDA-approved dose) subcutaneously twice daily for 6 months (26). However, despite the fact that B and T cells were mobilized to blood, and that naive B cells underwent class switching in vitro, there was not an improvement in Ig levels or vaccine responses (26). Thus it appears that alternate dosing regimens or a more long-lasting CXCR4 antagonist will be required to achieve more durable activity that may in turn provide more stable levels of immune cells in the blood to enhance adaptive immune function.

CXCR4 activating mutations have also been recently found in Waldenstrom macroglobulinemia (WM). WM is an incurable B-cell neoplasm characterized by accumulation of malignant lymphoplasmacytic cells (LPC) in the bone marrow (BM), lymph nodes, and spleen, with excess production of serum immunoglobulin-M (IgM) producing hyperviscosity, tissue
infiltration and autoimmune-related pathology. Activating somatic mutations have been found by whole genome sequencing in WM, including a locus in CXCR4 (27). In addition, approximately 90% to 95% of WM patients harbor an activating mutation in MYD88 (MYD88<sup>L265P</sup>) that promotes both the interleukin-1 receptor–associated kinase (IRAK) and Bruton’s tyrosine kinase (BTK), which in turn activate nuclear factor-kB-p65–dependent nuclear translocation and malignant cell growth. The location of the CXCR4 somatic mutations in the C-terminal domain of WM patients is similar to the location of the mutations observed in the germ line of patients with WHIM syndrome. Somatic mutations in MYD88 and CXCR4 are thus felt to be important and to impact overall survival in WM (27), opening the way for possible combination therapy directed against MYD88 and CXCR4 signaling in WM (27).

The CXCR4-CXCL12 axis as a potential target in cancer therapy

While there has been great enthusiasm for exploiting the CXCR4-CXCL12 axis as a target in cancer therapy, to date the promise has yet to be fulfilled. Initial interest in pursuing the CXCR4-CXCL12 axis as a target for cancer therapy was fueled by observations implicating CXCR4 in promoting metastasis (2, 28). CXCR4 is expressed in at least 20 different human cancers (8, 29-33); CXCR7 is also expressed in tumors and found to be involved in cell growth, survival, and metastasis (34, 35). Like CXCR4, it is expressed on tumor-associated vessels and on neovasculature (36). On the other hand, CXCL12 is secreted in the tumor microenvironment by stromal cells (21, 28). Based on data such as these, it has been thought that CXCR4 antagonism could prevent the development of metastases by targeting multiple steps in the process of dissemination. Restricted by the tumor type, inhibiting CXCR4 should interfere with tumor cell growth, migration and chemotaxis, and homing toward secondary organs.

In addition to a potential role for agents targeting the CXCR4-CXCL12 axis in preventing metastasis, other indications have been suggested. Some have proposed that inhibition of the
CXCR4-CXCL12 axis could be used to mobilize leukemic cells from the marrow, rendering them more amenable to cytotoxic chemotherapy by removing them from the pro-survival stem cell niche (8, 21, 37-39).

Others have suggested that modulation of the CXCR4-CXCL12 axis could revert the tolerogenic polarization of the microenvironment rich of immunosuppressive cells such as Treg, M2 and N2 neutrophils (40-42). Recent data show that blocking the interaction of T-cells expressing CXCR4 with cells in the microenvironment secreting CXCL12 may modulate immunotherapy with anti-CTLA4 or anti-PD-1 (41, 42). Although inhibition checkpoints have been shown to induce immune-mediated tumor shrinkage, major responses have been reported in only a subset of patients following PD-1 blockade (43). The resistance to immunotherapy reported in colon, ovary cancer and in the model of pancreatic ductal adenocarcinoma (PDA) is based on multiple mechanisms: spatial distribution of effector T cells relatively to tumor, recruitment of tumor specific T cells from the vessel, and T cells proliferation activity. In the PDA model, cancer associated fibroblasts (CAF) cells may regulate the T cell access to the tumor through release of CXCL12 which is bound by the PDA cancer cells. Plerixafor, inhibiting CXCR4, increases accumulation of T cells among cancer cells impairing tumor growth and increasing tumor sensitivity to anti-PD-L1. The related mechanism must involve either T cells or myelomonocytic cells, CXCR4 expressing cells. Of note, in this model neither cancer cells nor CAF-FAP+ cells express CXCR4 (41, 44). CXCR4-dependent modulation of immunotherapy was demonstrated in hepatocellular carcinoma (HCC) models. Sorafenib treatment of orthotopic HCC induced hypoxia responsible of development of resistance. Sorafenib resistance was mediated by increased presence of immunosuppressive infiltrates (F4/80, tumor associated macrophages (TAMs), myeloid derived suppressive cells and Treg) and increased PD-L1 expression in multiple hematopoietic cells, including immunosuppressive cells. Since the recruitment of
immunosuppressive cell population was partially mediated by CXCL12-induced hypoxia, the efficacy of CXCR4 inhibition was evaluated. A combined treatment (sorafenib/Plerixafor/anti-PD-1) showed the most pronounced delay in tumor growth probably mediated by increased intratumoral penetration and activation of CD8 T lymphocytes in HCC (42). Moreover, in mouse model of intraperitoneal papillary epithelial ovarian cancer, CXCR4 antagonism increased tumor cell apoptosis and necrosis, reduced intraperitoneal dissemination, and selectively reduced intratumoral FoxP3+ Treg cells (40). Consistently with these data, Plerixafor and the antagonistic CXCR4 peptide R (45, 46) inhibit Treg suppressive activity in primary renal cancer specimens in which higher numbers of activated Tregs (expressing CTLA-4/CXCR-4/PD-1/ICOS) were detected. A possible mechanism may involve the Treg-FOXP3 promoter.

Treg-FOXP3 activity is regulated post-translationally by histone/protein acetyltransferases and histone/protein deacetylases (HDACs). Pan-histone/protein deacetylase inhibitors (pan-HDACi) have been shown to increase FOXP3 acetylation and DNA binding, enhance Treg production and suppressive activity, and have beneficial effects in the prevention and treatment of autoimmune disease and transplant rejection (47). Since CXCR4 signaling transduces also on FOXP3 and HDACis upregulated CXCR4 mRNA expression (48) it is possible that CXCR4 modulation affects the acetylation status of FOXP3 promoter.

**CXCR4 antagonists in development**

Plerixafor has many positive attributes, including demonstrated anti-metastatic potential in preclinical studies; however, there is room for the development of additional agents targeting the CXCR4-CXCL12 axis. CXCR4 inhibition was originally conceived as a strategy to block infection of CD4+ T cells by HIV since CXCR4 functions as a co-receptor for T-tropic (X4) HIV virus entry (20). During development as anti-HIV drug, plerixafor displayed a lack of oral bioavailability and cardiotoxicity. Additionally, its toxicity profile is such that it limits long-term administration as
might be required for metastasis prevention therapy (49). Although a 6 months administration was tolerated in WHIM patients, doses utilized were 4% to 8% of the FDA-approved dose (26). Finally, plerixafor lacks CXCR4 specificity since it also binds the other CXCL12 receptor, CXCR7, as an allosteric agonist (50). The ability of CXCR4 antagonists to induce stem cell mobilization raised questions whether the same treatment might mobilize cells from solid tumors that express CXCR4. In a recent Phase 1 Study, the efficacy of the peptidic CXCR4 antagonist LY2510924 was concomitantly evaluated on CD34+ mobilization versus count of circulating tumor cells (CTC). Although there was significant mobilization of CD34+ cells upon treatment with LY2510924, no apparent effect on CTC count was observed (51).

Table 1 lists CXCR4 antagonists in clinical development. The majority of clinical trials have focused on the mobilizing activity of CXCR4 antagonists and were conducted in multiple myeloma, acute myeloid leukemia and chronic lymphatic leukemia (Table 1). Among peptidic inhibitors, 4F-benzoyl-TN14003 (BKT-140) is a 14-residue bio-stable synthetic peptide, which binds CXCR4 with a greater affinity compared with plerixafor. Studies in mice demonstrated the efficient and superior mobilization and transplantation of stem cells collected with G-CSF-BKT140, compared with the single agent alone (52, 53). Additional studies demonstrated anticancer activity (54) and an ability of BKT140 to directly induce cell death of chronic myeloid cells (CML) and to revert resistance to imatinib mediated by increasing BCL6 (37). A Phase I trial conducted in multiple myeloma patients showed that BKT140 was well-tolerated, and produced a robust mobilization that resulted in the collection of functional CD34+ cells which rapidly engrafted (55). However, to date, anti-cancer activity has not been demonstrated.

Evaluation of antimetastatic activity in solid tumors is in Phase I - II clinical development and a limited number of results are available. LY2510924 (Eli Lilly and Company®) is a potent selective peptide CXCR4 antagonist; a phase I trial showed that the most common drug-related
adverse events were fatigue, injection-site reaction and nausea. However its potential as an anti-cancer agent has yet to be established. In the phase I trial, the best response achieved was stable disease in nine patients (20%) (51). In two Phase II studies, one in renal cell carcinoma [NCT01391130] and one in small cell lung cancer (SCLC) [NCT01439568] that evaluated the efficacy of LY2510924 in combination with sunitinib and carboplatin/etoposide, respectively, anti-tumor efficacy was not achieved. In the SCLC trial there was no improvement in outcome for chemotherapy-naïve patients with extensive stage disease - small cell lung cancer (ED-SCLC) when 20 mg LY2510924 was added to carboplatin and etoposide (56). This outcome was observed despite preclinical data that was supportive of an anti-tumor strategy. The latter included 1) the fact that SCLC cells have been shown to express high levels of CXCR4 and activation of CXCR4 could induce cell migration and adhesion to stromal cells that secrete CXCL12, and this in turn could provide growth and drug resistance signals to the tumor cells; and 2) a previous study that reported CXCR4 antagonists disrupt the CXCR4-mediated adhesion of SCLC cells to stromal cells, in turn sensitizing SCLC cells to cytotoxic drugs such as etoposide, by antagonizing cell adhesion-mediated drug resistance (39). ED-SCLC may not be an ideal target for development as the disease often presents with extensive primary disease and with metastases at diagnosis and one would expect a CXCR4 antagonist to be mostly active in primary steps of metastatic dissemination.

CTCE-9908, a CXCL12 N-terminus derived peptide that is a dimerized sequence of CXCL12 amino acids 1-8 has also undergone a phase I-II clinical trial (57). CTCE-9908 was well tolerated with the most common side effect reported being mild to moderate injection site irritation in the 5.0 mg/kg/day dose group. However, the magnitude of anti-tumor efficacy was at best very modest, with six patients (30%) with evaluable although not clinically meaningful stable disease after one cycle (one month) (58).
Finally, we have recently reported a new family of CXCR4 antagonists developed through a ligand-based approach (45, 46). A three-residue segment identified in CXCL12 that was similar to, but in reverse order, to a peculiar inhibitory chemokine secreted by herpes virus 8 (HHV8) vMIP-II was the starting point (59, 60). The motif Ar1-Ar2-R, where Ar is an aromatic residue, constitutes the core of a cyclic peptide library evaluated for anti-CXCR4 activity. Three peptides (R, S and I) identified as potent CXCR4 antagonists were demonstrated to reduce the development of metastases in in vivo models (45, 46). Recent studies also demonstrated that peptides R, S and I efficiently mobilize LY6G+ cells better and longer than AMD3100 and increase bone marrow cellularity, mainly of immature precursors indicating an active hematopoietic stimulation.

The question is how best to design trials that will circumvent the problem that CXCR4 antagonists may not reduce primary tumor growth in cancers such as ovarian and colorectal cancer, where both local and distant metastasis prevention would be important and where CXCR4 likely plays a role. One proposed model of clinical development would be to add it to chemotherapy, such as fluorouracil and oxaliplatin, which are used in neoadjuvant treatment of locally advanced rectal cancer. Such a setting identifies a time limited treatment and a rapid evaluation of results. Moreover another ideal setting could be represented by treatment of glioblastoma following primary surgery in which it is possible hypothesize that CXCR4 inhibition coupled to radiotherapy may have an additional effect on vasculogenesis ameliorating tumor control, as shown in preclinical studies (61).

Conclusion

Extensive pre-clinical data indicates that targeting the CXCR4-CXCL12 axis may have several beneficial actions including: 1) affecting CXCR4-expressing primary tumor cells; 2) synergizing with other cancer-targeted therapies; and 3) modulating the immune response. While any or all of these could be valuable anti-cancer strategies, clinical results to date have been disappointing.
Additional studies and studies with new agents will be required to determine whether inhibition of the CXCR4-CXCL12 axis may produce enough anticancer activity to be effective therapy in the complex setting of human cancer.

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References

dissociation of CXCR4 activation from binding and inhibition of HIV-1. EMBO J 1997;16:6996-7007.


Table 1. CXCR4 antagonists under clinical investigation

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Figure 1. CXCR4-CXCL12-CXCR7 transduction pathway. CXCL12 acts on two distinct receptors, CXCR4 and CXCR7, which are 7 membrane transmembrane receptors/G protein coupled (GPCRs). CXCR4 and CXCR7 can form homodimers or heterodimers. CXCL12 shares CXCR7 binding with another CK, CXCL11 that is also a ligand for CXCR3. CXCR4 triggers preferentially G-protein-coupled signaling, whereas activation of CXCR7 or the CXCR4/CXCR7 complex induces β-arrestin-mediated signaling. The Gαi monomer inhibits the adenylyl cyclase activity regulating cell survival, proliferation and chemotaxis. While Gαi triggers PI3K/AKT/mTOR and ERK1/2, Gβγ dimer triggers intracellular calcium mobilization through phospholipase C (PLC). When CXCL12 binds CXCR7, the receptor signals through β-arrestin, inhibits G-protein-coupled signaling and activates MAP kinase pathway. CXCR7 can also signal through PLC/MAPK to increase cell survival. CXCR4–CXCR7 heterodimers-β-arrestin pathway can be activated through GPCR kinase (GRK)-dependent phosphorylation to internalize CXCR4, scavenging CXCL12 or/and control cell survival through ERK1/2. CXCL12 also causes CXCR4 desensitization, uncoupling from G-protein by GRK-dependent phosphorylation and β-arrestin-dependent endocytosis. In contrast to CXCR4, when CXCL12 binds CXCR7 the interaction between β-arrestin and CXCR7 internalizes the receptor, and subsequent recycles it to the cell membrane. Upon binding to CXCR4 or CXCR7, CXCL12 is internalized and subjected to lysosomal degradation. Activator signals _____; Inhibitory signals . . . . . .
Figure 1:

CXCL12

CXCR3

CXCR4

CXCR4/7

CXCL11

CXCR7

GRK

β-arrestin

PLC

ERK1/2

Ca²⁺

IP3

Lysosome

CXCL12 scavenging

Cell survival, proliferation

CXCL12 scavenging

CXCR4 endocytosis, desensitization

Migration, chemotaxis

Cell survival, proliferation

Proliferation, chemotaxis

Proliferation, chemotaxis

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